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16. ABSTRACT

This paper discusses the influence of the diet of test organisms upon the results of aquatic toxicity tests. The adequacy of Artemia nauplii generated from Reference Artemia Cysts II (RAC II) for tests with several species is detailed. Consideration is given to both nutritional adequacy and food contamination.

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STANDARDIZATION OF THE NUTRITION OF FISH IN AQUATIC TOXICOLOGICAL TESTING[1]

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I. TOXICITY TESTS: A BRIEF OVERVIEW

In the past few decades, increased public concern with environmental pollution has led to legislation and the formation of government agencies to regulate the addition of contaminants to the environment. Research is required to provide the scientific data that support the agencies' regulatory activities. One very important aspect of this research is toxicity testing of both individual chemicals and complex effluents using living organisms. The testing is done not only by government laboratories, but also by industrial and contract laboratories. In a toxicity test, groups of organisms are exposed to different toxicant concentrations and their responses (mortality, growth, reproduction, behaviour, etc.) are

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observed, quantified and compared with responses of organisms in a control treatment, not exposed to the toxicant. Toxicity tests can be generally subdivided into two categories, short-term, or acute, tests and long-term, or chronic, tests.

Historically, most toxicity tests conducted with aquatic organisms have been acute 48-h or 96-h exposures to a single chemical. The toxicant concentration necessary to kill 50% of the organisms (LC50) was calculated and provided a relative measure of the toxic properties of the chemical. Many of the tests were conducted with juvenile or adult organisms under static conditions. Feeding of the test organisms was prohibited not only during the test, but also for two days prior to it, in order to ensure the physiological similarity of the test organisms and to prevent fouling of the water. The philosophy and protocol of not feeding organisms during acute tests is still maintained (ASTM, 1980), even though the emphasis has switched to flow-through tests. Thus, consideration of the nutrition of the test organisms has been limited to ensuring that test organisms are not manifestly diseased or abnormal at the start of the test.

More recently, researchers have observed that larval organisms are often more sensitive to toxicants than their juvenile or adult stages and have also realized the importance of long-term sublethal toxicant effects. Toxicity testing has evolved to the point that longer term tests and tests utilizing larval organisms require that the organisms be fed during the test. One example of this evolution is the fish early life-stage (ELS) test (see, e.g., Woltering, 1984). ELS tests usually begin with 24-h old embryos that are placed in several toxicant concentrations plus a control. The test includes embryonic development, hatching, larval development and metamorphosis to the juvenile stage and can last from 28 to 120 days depending on species. Both survival and growth of the fish (i.e. both lethal and sublethal effects) are recorded. (In life-cycle tests with fishes and invertebrates, reproduction is measured in addition to survival and growth).

In order to ensure the interlaboratory comparability and legal defensibility of toxicity test data, standard methods for their conduct are being developed under the auspices of the American Society for Testing and Materials (ASTM) and the Organization for Economic Cooperation and Development (OECD). In the draft documents presently under consideration, it is recommended that fish in an ELS

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test be fed brine shrimp nauplii (Artemia), rotifers, or commercially available prepared diets, depending on the fish species used. Available evidence, some of which is presented below, shows that diet can significantly affect the results of toxicity tests and that such broad recommendations may be insufficent to ensure standardization of toxicity tests.

II. EFFECTS OF NUTRITION IN TOXICOLOGICAL TESTING

The Committee on Laboratory Animal Diets, in a report to the U.S. National Academy of Sciences (NRC, 1978), stated that: "The role of animal nutrition as an experimental variable has been overlooked for too long. Adequate, controlled nutrition for laboratory animals is essential to the reproducibility of data, and in the long run it results in a net saving of time and money. The Committee believes that few if any other factors can influence the results of a study as profoundly as diet." Although written with regard to mammalian species, their conclusions apply to fish species as well.

Mehrle et al. (1974) fed six prepared diets to separate groups of juvenile rainbow trout (Salmo gairdneri) for six weeks prior to their use in a 96-h acute toxicity test with chlordane. The LC50 values for the groups of fish varied more than five-fold, from 8.2 μg/l to 47 μg/l. In particular, the fish fed a synthetic low-protein diet were significantly more sensitive than fish fed a high protein diet. Mehrle et al. (1977) later found that a) when juvenile rainbow trout and bluegills (Lepomis macrochirus) were fed two different diets during a 30-day chronic toxicity test, one diet caused both species to be more sensitive to the PCB Arochlor 1254 than did the other diet; b) dietary methionine concentration caused significant differences in the toxicity of DDT and dieldrin to adult rainbow trout in a chronic test; c) increased vitamin C in the diet of juvenile channel catfish (Ictalurus punctatus) increased their tolerance in a chronic test with toxaphene.

Several other studies, both short- and long-term, have been conducted on dietary effects in toxicological tests with rainbow trout. Phillips and Buhler (1979) fed fingerlings on tubificid worms or a commercial pelleted diet during chronic exposure to a sublethal concentration

of dieldrin. The fish fed tubificids had reduced fat elaboration and reduced body concentrations of dieldrin than the fish fed pellets. Yamamoto et al. (1981) showed that increased dietary L-ascorbic acid resulted in decreased copper uptake in an 18-week chronic exposure to that metal. Increased carbohydrate content in the diet resulted in nearly a 2-fold increase in copper toxicity in a short-term test (Dixon and Hilton, 1981). Two commercial diets led to significantly different hepatic enzyme responses of trout exposed to carbon tetrachloride for 24 h (Pfeifer et al., 1980).

Even when fish are fed live Artemia nauplii from different geographical sources, toxicity test results can be significantly altered. Bengtson et al. (in press) found that acute toxicity of cadmium to 25-day old Atlantic silversides (Menidia menidia) differed by 2 1/2 times, depending on which of two geographical strains of Artemia the fish had been fed prior to testing. Schimmel (1981) has pointed out that intralaboratory variability for repeated determinations of LC 50 for a single species is generally less than a factor of two; interlaboratory variability should be less than a factor of four.

The effects of nutrition on aquatic toxicity testing are not limited to fish, but have also been demonstrated for several crustaceans, among them cladocerans (Winner et al., 1977; Taylor, in press), copepods (Sosnowski et al., 1979; Cooney et al., 1983) and mysids (Bengtson et al., in press).

In some of the studies just cited, diets were of known composition and were purposely caused to vary. In others, diet composition was unknown. In either case, however, two aspects of the diet are important with regard to toxicity testing: nutrient levels and contaminant levels.

Specific nutrient requirements are known for relatively few species of fish (e.g., some trout and salmon species, channel catfish, carp) and are unknown for many species commonly used in toxicity tests, e.g. fathead minnows (Pimephales promelas), sheepshead minnows (Cyprinodon variegatus), silversides (Menidia spp.). For commercially available formulated diets, the proximate composition is very likely the only available nutritional information on the package. For commercially available Artemia cysts, it is unlikely that any nutritional information is available. Thus, the toxicologist knows almost nothing about his test organisms' nutrition unless he analyzes batches of diets himself.

III. THE NUTRITIONAL VARIABILITY OF DIETS

Literature reports of analyses of variability in nutrient levels in commercially available products are not encouraging. Crawford and Law (1972) and Tacon and DeSylva (1983) found that mineral composition was highly variable in the diets they tested. Fatty acid composition of *Artemia* has been shown to vary significantly with geographical source (Watanabe *et al.*, 1978b; Fujita *et al.*, 1980; Schauer *et al.*, 1980; Seidel *et al.*, 1982) and that of rotifers (*Brachionus plicatilis*) has been shown to vary with culture conditions (Watanabe *et al.*, 1978a).

Contaminant levels in commercial fish feeds have been analyzed and the results again indicate great variability. Brauhn and Schoettger (1975) found DDT levels ranging from nondetectable to 390 ppb and PCB levels from nondetectable to 320 ppb in the diets they analyzed. Fish oils used in those diets had the highest concentration of organic contaminants of all the components used. Crockett *et al.* (1975) detected DDTs of 20 to 840 ppb and lesser levels of five other organic contaminants in catfish feed. Gruger *et al.* (1976) measured 110 ppb total chlorobiphenyls in the diet used for a study of sublethal effects of chlorobiphenyls in salmon. They pointed out the need that researchers have for contaminant-free fish food, but acknowledged that the development of a clean diet without nutritional deficiencies would take years. Parejko and Wu (1977) analyzed two commercial diets fed to hatchery trout and found heptachlor levels of 21 and 15 ppb, DDE levels of 260 and 200 ppb, and total Arochlor levels of 118 and 639 ppb. Mac *et al.* (1979) found PCB concentrations from 100 to 300 ppb and DDE concentrations from undetectable to 470 ppb in different lots of one commercial fish feed. They also found 26 ppb DDE and 140 ppb PCB in brine shrimp nauplii and suggested that the nauplii should not be considered as an alternative feed to avoid contamination. They concluded that "the presence of organic contaminants in fish feed demonstrated in this and other studies is evidence that all feed used in contaminant-exposure studies should be analyzed."

The contamination of *Artemia* nauplii by chlorinated hydrocarbons and heavy metals has also been shown to vary tremendously with geographical source of the *Artemia*. The results of Olney *et al.* (1980) and Seidel *et al.* (1982) indicated that total DDTs ranged from 2 to 422 ppb and total PCBs ranged from 1 to 66 ppb depending on source.

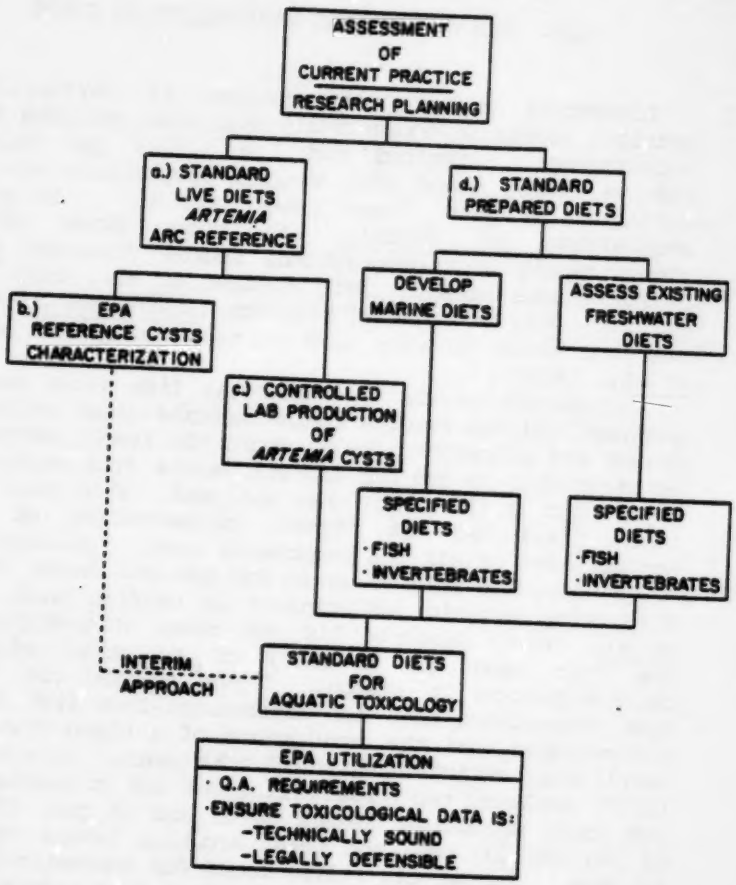

Fig. 1. Research strategy for nutrition in aquatic toxicology.

Considerable information exists, therefore, to show the large amount of variability of both nutrients and contaminants in both living and prepared diets for fish. Two major questions that remain, however, are: at what concentrations do dietary contaminants affect the biology of the fish?; and at what concentrations do dietary contaminants interact with toxicant effects in toxicity tests? Present ASTM (1980) recommendations for maximum contaminant levels in food for toxicity test organisms are < 150 ppb organic chlorine or < 300 ppb total

organochlorine pesticides plus PCBs.

Mammalian toxicologists have long understood the importance of nutrition of animals prior to and during testing (NRC 1976, 1978). Certified diets are now available (Shelton, 1980), in which certain contaminants are certified not to exceed specific minimum levels. Greenman et al. (1980) and Bercz (1981) have reported on unacceptable contaminant levels in uncertified mammalian diets and have given examples of toxicity test data that had to be discarded for that reason.

The use of standard certified diets should be adopted by aquatic toxicologists as well, in order to reduce a potentially serious source of experimental variability. Our research strategy toward the accomplishment of that goal for the U.S. Environmental Protection Agency is outlined in Fig. 1. Standardization of both live and prepared diets is necessary. We have so far completed the interim approach (standardization of Artemia) and are just beginning work on the development of prepared diets for marine larval fish and the certification of existing diets for freshwater fish.

IV. STANDARIZATION OF BRINE SHRIMP NAUPLII

The concept of a standard, or reference, lot of brine shrimp cysts for use in scientific research was originally proposed (Simpson et al., 1980) in response to the variability in nutritional quality of different geographical strains of Artemia cysts (see literature reviewed in Beck and Bengtson (1982) or Seidel et al. (1982)). The availability of a standard lot, referred to as Reference Artemia Cysts (RAC), was announced to the scientific community (Sorgeloos, 1980) and samples have been sent to scores of scientists around the world.

Because of the importance of Artemia nauplii as a food for fish in ELS tests (as well as for several invertebrate species), the U.S. EPA has acquired and tested a large homogeneous lot of brine shrimp cysts, designated RAC II, as a standard diet for marine organisms used by EPA in toxicity testing. Testing of RAC II included several biochemical analyses and evaluation as a diet for two fish and two crustacean species, following methods of Beck et al. (1980), Johns et al. (1980, 1981), Klein-MacPhee et al. (1980), Olney et al. (1980), Schauer et al. (1980), and Seidel et al. (1980). Analysis for chlorinated hydrocarbons (Table I) indicated that RAC II was

Table I. Chlorinated hydrocarbons in Reference Artemia Cysts II (RAC II) nauplii. Results are given as ng/g or ppb.

Chlorinated Hydrocarbon	Concentration
HCB	< 0.1
α -BHC	< 0.4
γ -BHC	< 0.6
cis-chlordane	< 0.5
trans-chlordane	< 0.6
nonachlor	< 0.3
dieldrin	< 1.4
Σ DDTs (ppDDE, ppDDD, opDDT, ppDDT)	< 10
Σ PCBs (1016 + 1254)	< 20

relatively free of contamination, with < 10 ppb total DDTs and < 20 ppb total PCBs. Fatty acid data (Table II) show that the level of the essential fatty acid 20:5ω3, while less than that for RAC and Brazilian samples, is certainly adequate for marine organisms (Schauer et al., 1980). Essential amino acid requirements (for chinook salmon) are also met by RAC II (Table III). Proximate and mineral analyses (Table IV) also suggest that RAC II may be a good larval fish diet.

Laboratory evaluation of the RAC II diet included its acceptability as measured by survival, growth and reproduction of species commonly used in toxicity tests. Fish larvae were fed RAC II, RAC (known good-quality diet), or San Pablo Bay (known poor-quality diet) and survival and growth were measured. The fish survived and grew as well on RAC II as on RAC (Table V). Evaluation as a diet for two crustacean species (Table VI) showed that RAC II allowed good survival to the megalops stage for mud crab larvae, which show a clear response to

Table II. Weight percent fatty acid composition of RAC II *Artemia* nauplii. Data from other high-quality *Artemia* nauplii are included for comparison (RAC data from Seidel *et al.* (1982), Brazil data from Schauer *et al.* (1980)). (nd = not detected).

FAME	RAC II	RAC	Brazil
14:0	1.93	1.79	1.57
15:0	0.50	nd	0.67
15:1	0.42	nd	0.24
16:0	14.81	12.70	15.42
16:1	16.82	16.78	10.79
16:2 ω 4	4.12	4.33	3.88
18:0	4.30	4.07	2.79
18:1 ω 9	34.82	30.37	35.86
18:2 ω 6	11.80	9.62	9.59
20:0	0.23	nd	0.52
20:3 ω 3/20:4 ω 6	5.16	5.82	nd
20:5 ω 3	5.14	8.45	8.98

inadequate diets by complete mortality at metamorphosis to megalops (Johns *et al.*, 1980), and provided good reproduction by mysids, also a good indicator of diet adequacy (Johns *et al.*, 1981).

We recommend that RAC II be used as a certified diet because it has been extensively characterized for its chemical constitutents and it has met biological requirements for species used in marine toxicity tests. The availability of this standard diet reduces the variable of nutrition of test organisms, which may reduce variations seen in interlaboratory comparison of data, and should improve the quality of the toxicity test data produced.

Table III. Amino acid profiles for RAC II *Artemia* nauplii. Data from Seidel *et al.* (1980) for another high-quality *Artemia* strain (Brazil) and the dietary essential amino acid requirements for chinook salmon (Mertz, 1969) are also listed for purposes of comparison. Values are expressed as g amino acid/ 100 g protein.

Amino Acid	RAC II	Brazil	Chinook Salmon
Aspartic acid	10.3	11.0	-
Threonine	5.5	5.2	2.5
Serine	5.1	4.5	-
Glutamic acid	14.1	13.1	-
Proline	6.9	5.7	-
Glycine	7.4	6.0	-
Alanine	7.8	4.6	-
Valine	6.8	5.3	3.25
Methionine	3.3	2.2	1.5
Isoleucine	5.9	5.6	2.5
Leucine	9.3	8.9	4.0
Tyrosine	4.7	10.5	-
Phenylalanine	4.6	5.1	5.25
Histidine	4.3	4.9	1.75
Lysine	10.3	11.7	5.0
Arginine	9.8	11.5	5.75

Table IV. Proximate analysis and mineral analysis of RAC II nauplii.

a) Proximate analysis (%)

	Dry			
Moisture	Protein	Fat	Fiber	Ash
82.4	47.0	20.8	5.2	6.1

b) Mineral analysis

Na %	1.86
K %	1.27
Mg %	0.15
P %	0.11
Ca ppm	303
Mn ppm	13.9
Zn ppm	118
Fe ppm	269
Cu ppm	15.5

This standard diet, however, is effective only as long as the supply lasts, estimated to be 3 years. After that, a new standard will have to be established. Reliance on Artemia cysts from natural populations presents several problems. Unpredictable climatic conditions, unreliable biological management of salinas (salt pans), and availability or cost of cysts in fluctuating world market conditions all mitigate against the likelihood that cysts can even be purchased from the same geographical source, let alone that they might be similar in quality.

The production of Artemia cysts under controlled laboratory conditions (Versichele and Sorgeloos, 1980; Lavens and Sorgeloos, in press) may provide a long-term solution to the problem of variability of Artemia from natural populations. The U.S. EPA is presently funding a portion of the research being done on lab cyst production

Table V. Survival and growth of two fish species, Atlantic silversides (Menidia menidia) and winter flounder (Pseudopleuronectes americanus) raised on nauplii of Reference Artemia II (RAC II), Reference Artemia (RAC) or San Pablo Bay Artemia (SPB). Silversides were reared for 21 days, flounder for 26 days, on different diets. Data are mean ± standard deviation.

	M. menidia		P. americanus	
Food	Survival (%)	Final wet wt. (mg)	Survival	Final wet wt. (mg)
RAC II	89.4 ± 5.9	16.8 ± 4.6	83.7 ± 6.3	2.60 ± 3.33
RAC	79.3 ± 6.8	19.3 ± 5.4	76.2 ± 9.5	1.55 ± 2.36
SPB	21.8 ± 5.2	19.3 ± 6.3	17.8 ± 14.2	0.60 ± 0.58

at the Artemia Reference Center in Ghent, Belgium. If cysts can be produced in sufficient quantity and quality under controlled conditions of feeding, temperature, water quality, etc., then reliance by toxicologists on natural Artemia populations could be curtailed.

V. RESEARCH NEEDS

Beyond the controlled laboratory production of Artemia cysts, standard nutrition of fish in toxicology requires research in three general areas. The first is the development of certified diets for species whose nutrient requirements are (at least partially) known and which are used for toxicity testing, e.g., rainbow trout and channel catfish. Progress has been made in this direction with the development of reference research diets at the U.S. Fish and Wildlife Service Laboratory at Columbia, Missouri (Brauhn and Schoettger, 1975). The second is the investigation of nutrient requirements of those species used in toxicology whose requirements are not known and the development of formulated diets for them. This aspect is especially applicable to marine fish larvae. Finally, we need to better determine the maximum levels of contaminants allowable in fish diets that are used in

toxicology.

The studies of Murai et al. (1981) and Hodson and Hilton (1983) are helpful in this regard. We especially need to detemine at what concentrations certain dietary contaminants have interaction effects with the toxicants being studied.

Table VI. a) Survival and growth of two crustacean species, mud crab (Rhithropanopeus harrisii) and mysid (Mysidopsis bahia) raised on nauplii of Reference Artemia II (RAC II), Reference Artemia (RAC), or San Pablo Bay Artemia (SPB). Mud crabs were raised to the megalops stage, mysids were raised for 10 days after hatching. b) Survival, growth and reproduction of mysids raised for 25 days (full life cycle). Survival is percentage of the original 35 mysids per replicate that survived to the end of the experiment, dry weight is the average of dry weights of those survivors and juvenile production is the number of juveniles produced per adult female at the end of the experiment. Data are means ± standard deviation.

a)

	R. harrisii		M. bahia	
Food	Survival(%)	Final dry wt. (ug)	Survival(%)	Final dry wt. (ug)
RAC II	78.6 ± 12.5	104 ± 26	73.6 ± 9.4	138 ± 30
RAC	78.3 ± 10.2	122 ± 34	70.0 ± 0.0	180 ± 57
SPB	0.0	--	48.0 ± 16.5	181 ± 47

b)

Food	Survival(%)	Dry weight (ug)	Juvenile production
RAC II	76.7 ± 0.0	518 ± 154	6.85 ± 1.06
RAC	56.7 ± 0.0	523 ± 145	5.00 ± 1.21
SPB	50.0 ± 9.4	459 ± 124	0.19 ± 0.04

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